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DETERMINATION OF NUCLEOPOLYHEDRON COUNTS AND SIZE FREQUENCY
DISTRIBUTIONS BY MEANS OF A COULTER TRANSDUCER^{1/}

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Abstract

A Coulter counter, model B, with upper and lower threshold discriminators and a 30-micron aperture tube, can be used for the enumeration of nucleopolyhedra in suspensions--either pure or with varying amounts of impurities; also can establish size frequency distributions of viral inclusion bodies. Procedures are described in detail and illustrated.

Introduction

Particle counts have been utilized as a basis for standardizing suspensions of inclusion bodies of nucleopolyhedrosis virus. In insect control experiments, the number of polyhedral inclusion bodies in a given weight or volume of a nucleopolyhedrosis virus preparation

^{1/} Mention of a proprietary name does not imply endorsement or approval of the product by the U.S. Department of Agriculture to the exclusion of others which may also be suitable.

is frequently used as an indicator of the potency of such preparation. For example, the two following units are in current use in the United States: viral unit (VU) = 1×10^9 nucleopolyhedra; and larval equivalent (LE) - 6 VU = 6×10^9 nucleopolyhedra (Allen 1967; Woodall and Ditman 1967).

Experts disagree as to the validity and reliability of polyhedron counts for the estimate of the activity of a viral preparation in lieu of biological assay (determination of the LD₅₀ or ID₅₀).^{2/} Our position is that the polyhedron count can be, at times, quite misleading, particularly when used for comparison of the potency of different batches of viral material. However, once a correlation has been established between polyhedron count and activity of a viral preparation, the count may be used quite satisfactorily in lieu of the LD₅₀ or ID₅₀, particularly when a rapid indirect titration method is needed (as under field conditions).

Our laboratory has adopted two counting procedures: One is microscopic, and the other is electronic and requires elaborate equipment. The microscopic procedure, with a counting chamber (hemacytometer), is simple and can be recommended for the occasional determination of polyhedron counts, particularly under field conditions. A rapid automated procedure, on the other hand, is very useful for the recurring determination of inclusion-body counts of large series of preparations in the laboratory. The automated procedure used in our laboratory, as well as its advantages and disadvantages, will be outlined. Particle-size frequency distribution curves of some typical preparations will be presented.

Materials and Methods

Nucleopolyhedron Suspensions

The suspensions of nucleopolyhedra used in this study were prepared from tissue homogenates of larvae of the Douglas-fir tussock moth (*Hemerocampa pseudotsugata*) which were inoculated with nucleopolyhedrosis virus. The tissues were finely ground in buffered saline^{3/} with the addition of 0.00075 percent sodium dioctyl

^{2/} LD₅₀ or ID₅₀ is the dose which produces a response in 50 percent of the test subjects. If the response is death the dose is called median lethal dose (its symbol is LD₅₀); if the response is progressive infection, the dose is called median infective dose (ID₅₀).

^{3/} NaCl, 9 grams; 2-amino-2-(hydroxymethyl)-1,3 propanediol with hydrochloride (Trizma 7.1), 1.558 grams; distilled H₂O, to 1,000 ml.; HCl, 0.1 N, as needed to adjust the pH at 7.0 (at 25° C).

sulfosuccinate, a surface active agent. These suspensions were cleaned by centrifugation in sucrose density gradient columns.

One series of six paired counts (particle counting chamber and Coulter counter) was performed with a suspension obtained by isopycnic density gradient centrifugation as described by Martignoni (1967). This suspension was very pure, as revealed by dark-field microscopic examination and by bioassay. The number of particles other than polyhedra was 2 percent of the gross count; i.e., of all counted particles in the size range of the inclusion bodies. The main purpose of this series of counts with a purified suspension was to test the agreement between electronic counts and counts performed with a microscope counting chamber.

A second series of six paired counts was performed with six different preparations containing varying amounts of impurities. The number of particles other than polyhedra varied between 1.5 and 7.9 percent of the gross count; i.e., of all counted particles in the size range of the inclusion bodies. These suspensions were prepared as follows: Tissue homogenates were floated on columns of sucrose solutions (650 and 720 milligrams per milliliter) and spun for 1 to 2 hours at 25,000 revolutions per minute (maximal gravity field 112,000 x gravity). The fraction at the interface between the two sucrose solutions was collected, rinsed, and appropriately diluted with buffered saline. The main purpose of this second series of counts was to test the accuracy of the method when applied to preparations containing varying amounts of impurities, both within and outside the size range of the inclusion bodies.

Microscopic Counting Procedure

All microscope counts were made with one Spencer Bright Line counting chamber with double improved Neubauer ruling (No. 1492, American Optical Company, Buffalo, N. Y.). The chamber was filled with the polyhedron suspension in accordance with routine blood-cell counting procedures. The filled chamber was placed on the stage of a phase contrast microscope equipped with a 16X objective. The inclusion bodies were allowed to settle for 5 minutes. Six replicate counts were made for each preparation: Each count covered an area of 5 square millimeters, in separate single chambers. Thus, the total counting area was 30 square millimeters. In no instance did the number of polyhedra per square millimeter exceed 50. The polyhedron count was computed as follows:

$$\text{Number of polyhedra per microliter of stock suspension} = \frac{\text{Total number of polyhedra counted} \times \text{dilution} \times 10}{30}$$

The counts were performed independently by two observers who were quite familiar with the appearance of nucleopolyhedra as seen under phase contrast. These two observers used their judgment in rejecting certain particles which did not appear to be nuclear polyhedra. This

decision was carried out rather frequently (and independently) when the suspension contained particulate impurities in the same size range as the viral inclusion bodies.

Electronic Transducer Counting Procedure

To enumerate polyhedra in a suspension which also contains other particles in the approximate size range of the polyhedral inclusions, the following procedure was developed: (1) determine the total number of particles of a size range encompassing the polyhedron size distribution (gross count); (2) dissolve the nucleopolyhedra by treatment with alkali and ultrasound and take a second count (insoluble particle count); (3) obtain the number of nucleopolyhedra in the suspension by subtraction of the insoluble particle count from the gross count (net count).

The instrument used was a Coulter counter, model B, with upper and lower threshold discriminators, an aperture tube with 30-micron orifice, and a sampling volume of 50 microliters. Operation of the model B counter is described in detail in the Coulter "Instruction and Service Manual" (Coulter Electronics, Inc. 1966). All instrument counts were corrected for background particles and coincidence loss. A coincidence correction chart for the 30-micron aperture was supplied by Coulter Electronics, Inc. Dilutions of the polyhedron suspensions were such that in no instance the number of polyhedra per 50-microliter sample exceeded 23,000. The instrument time required for each count (50-microliter sample) was 14 seconds. Ten repeat counts in pairs of alternating polarity were taken for each preparation.

For counting with the electronic transducer, the suspensions were prepared with the following buffered saline:

NaCl	9 grams
2-amino-2-(hydroxymethyl)- 1,3 propanediol with hydrochloride (Trizma 7.1)	1.558 grams
Distilled H ₂ O	to 1,000 milliliter
HCl, 0.1 N	as needed to adjust the pH at 7.0 (at 25° C.)

Before use, the conductive solution was filtered through GS membranes (pore size 0.22 micron; Millipore Filter Corp., Bedford, Mass.).

The following formula was used for computation of the net nucleopolyhedron count (*C*):

$$C = (\alpha - \alpha_{bp} + \alpha_{cl}) - (b - b_{bp} + b_{cl})$$

where α is the gross count, b the insoluble particle count, α_{bp} and b_{bp} the respective background particle counts, α_{cl} and b_{cl} the respective coincidence losses. A step-by-step procedure for the determination of these quantities follows.

1. Rinse a clean 100-milliliter beaker with distilled water and pour about 80 milliliters of buffered saline in it. Place on platform in sample stand and stir.
2. Make five pairs of 50-microliter counts. Stop stirrer during each counting period. Compute and record mean count. This is the background particle count, α_{bp} .
3. Measure 49 milliliters of the saline and return it to the same beaker, after discarding the surplus.
4. To the 49 milliliters of saline add 1 milliliter of the test suspension of nucleopolyhedra, diluted to provide an instrument count between 5,000 and 25,000 per 50-microliter sample. Place on platform in sample stand and stir.
5. Make five pairs of 50-microliter counts. Stop stirrer during each counting period. Compute mean count. This is α .
6. Read the coincidence correction factor in the Coulter coincidence correction chart (for 30-micron apertures). (This factor varies between 0.4 and 2.1 percent for instrument counts between 5,000 and 25,000.) Multiply the instrument count by this factor. This is α_{cl} .
7. Place 1 milliliter of the test suspension of nucleopolyhedra (the same as used in step 4) in a Kolmer tube.
8. Add 1 milliliter of an alkaline solution prepared by mixing equal volumes of 0.2 molar NaOH and 0.1 molar Na_2CO_3 + 0.2 molar NaCl. [Both stock solutions are made freshly every second week and filtered through HA membranes--pore size 0.45 micron; Millipore Filter Corp.] Cover the tube with a plastic membrane and let stand for 5 minutes at room temperature with occasional agitation.
9. Expose the tube to ultrasound by immersing it for 10 minutes in the tank of an ultrasound generator (80 kilocycles per second at 80 watts).
10. Make a background count for the saline (as in step 2) and record. This is b_{bp} .

11. Measure 48 milliliters of this saline and return it to the same beaker, after discarding the surplus.
12. To this volume of saline add the 2 milliliters of treated suspension (from step 9). Place on platform in sample stand and stir.
13. Make five pairs of 50-microliter counts. Stop stirrer during each counting period. Record mean count. This is b .
14. Determine b_{c1} as in step 6.
15. Determine C , using the equation outlined above.

For very clean nucleopolyhedron suspensions--as obtained by repeated isopycnic density gradient fractionation (Martignoni 1967)-- b is equal or very close to b_{bp} and, consequently, b_{c1} is equal or very close to zero. Thus, steps 7 to 14 inclusive can be omitted, and

$$C = \alpha - \alpha_{bp} + \alpha_{c1}.$$

Considerable time can be saved by use of a flow-through cuvette (Bull 1967), particularly if many suspensions have to be handled in a relatively short time interval.

Determination of Size Frequency Distributions

Size distribution data were obtained from very clean suspensions of nucleopolyhedra in accordance with procedures described in the Coulter "Instruction and Service Manual" (Coulter Electronics, Inc. 1966). The upper threshold mode switch was placed in the "locked" position, the upper threshold dial was set at 50, and successive counts were taken at lower threshold intervals of five divisions. With such procedure, 20 windows of five dial divisions each are obtained. Calibration of the Coulter counter was performed with monodisperse polystyrene latex spheres (diameter, 2.96 microns; Dow Chemical Co., Midland, Mich.).

Results and Discussion

Polyhedron Counts

Tables 1 and 2 summarize the results of the two series of paired counts.

The relation between the two variables (microscopic counts and electronic counts) was measured by means of the coefficient of correlation. For the purified preparation, $r = 0.9997$, and for the series of impure preparations, $r = 0.9947$. Both coefficients are highly significant, and thus, we can state that the two variables are very closely related. This is evident also from an inspection of figures 1 and 2.

Table 1.--Results of one series of paired counts (microscopic and electronic) with a purified nucleopolyhedron suspension^{1/}

Count number	Microscopic count		Electronic count	
	Number of polyhedra counted	Number of polyhedra per microliter	Number of polyhedra counted	Number of polyhedra per microliter
1	327	5,450	54,480	5,448
2	589	9,817	100,248	10,025
3	818	13,633	137,170	13,717
4	1,022	17,030	166,942	16,694
5	1,187	19,783	195,787	19,579
6	1,304	21,733	215,450	21,545

^{1/} Microscopic counts are the average of six counts, each of 5 square millimeters. Electronic counts are the average of 10 repeat counts in pairs of alternating polarity.

Table 2.--Results of one series of paired counts (microscopic and electronic) with six impure nucleopolyhedron suspensions^{1/}

Count number	Microscopic count		Electronic count	
	Number of polyhedra counted	Number of polyhedra per microliter	Number of polyhedra counted	Number of polyhedra per microliter
1	450	7,500	75,127	7,513
2	508	8,467	83,545	8,355
3	685	11,417	113,858	11,386
4	697	14,463	118,458	14,748
5	1,118	18,633	174,958	17,496
6	1,261	21,017	215,320	21,532

^{1/} Microscopic counts are the average of six counts, each of 5 square millimeters. Electronic counts are the average of 10 repeat counts in pairs of alternating polarity.

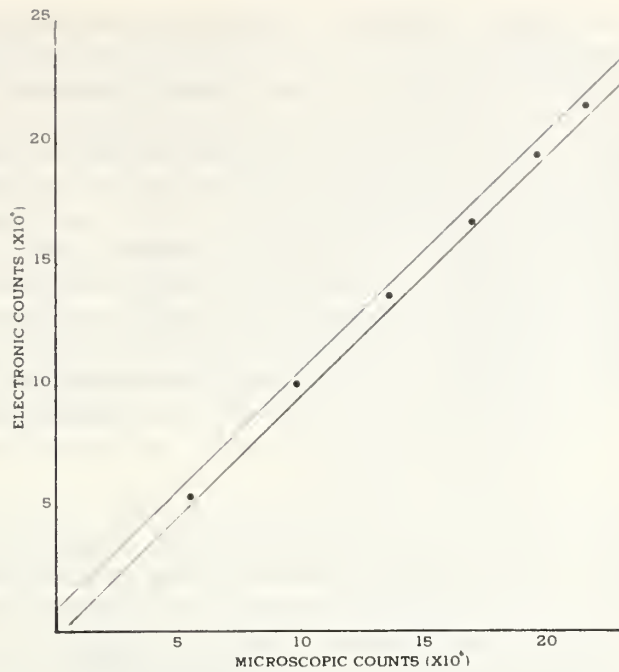


Figure 1.--Scatter diagram of microscopic counts of nucleopolyhedra in purified suspensions, plotted against electronic counts, with 0.95 confidence limits.

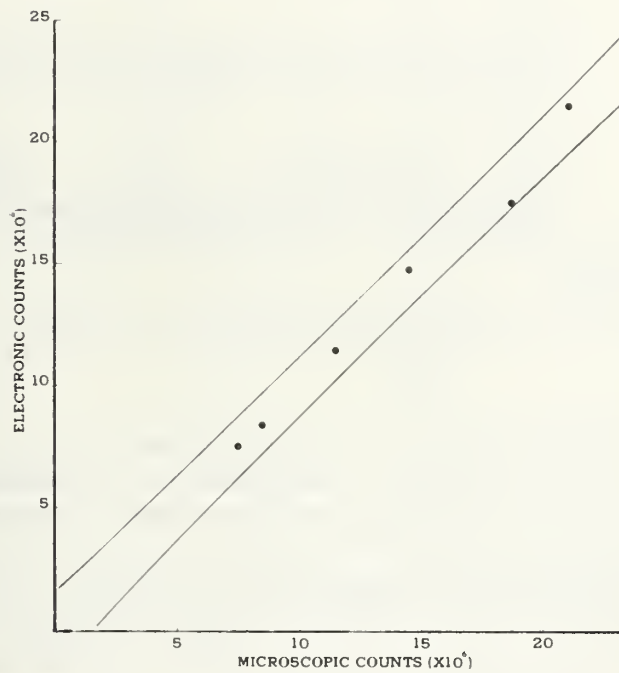


Figure 2.--Scatter diagram of microscopic counts of nucleopolyhedra in six impure preparations, plotted against electronic counts, with 0.95 confidence limits.

There is some slight evidence to indicate less agreement between the two counting methods in the case of the impure suspensions. A *t* test for the difference between the two coefficients of correlation indicates significance at the 10-percent probability level but not at the 5-percent level. This slightly lower correlation between the two methods in the case of the impure preparations may be attributable to the presence of alkali-soluble impurities in the nucleopolyhedra size range and of particles similar to nucleopolyhedra when observed in the microscopic counting chamber. However, for all practical purposes, the electronic method can be considered at least as reliable as the microscopic method, even when the polyhedron suspensions are relatively impure. The electronic method has the advantage of being independent from the decisionmaking process of an observer skilled in the differentiation of nucleopolyhedrosis virus inclusion bodies from other similar particles of nonviral origin.

Tables 1 and 2 also illustrate one of the important advantages of an electronic transducer for particle-counting procedures. The electronic counts were based on actual numbers of counted particles which were approximately 170 times greater than the number of particles counted with the conventional microscopic procedure. The percent standard error of the microscopic counts (which fit a Poisson distribution) is thus considerably reduced by the electronic counting procedure, as shown by the two examples in table 3.

Table 3.--*A comparison of the standard errors of two sets of paired counts (examples from table 2)*

Quantities	Count no. 1		Count no. 6	
	Microscopic	Electronic	Microscopic	Electronic
Mean count, polyhedra per microliter	7,500	7,513	21,017	21,532
Standard error, polyhedra	353	27	593	47
Standard error, percent of mean	4.71	0.36	2.82	0.22

When the suspensions are prepared with raw tissue homogenates, the agreement between the two counting procedures is erratic and, in general, very poor. Electronic counts are usually higher than microscopic counts, possibly because the trained microscopist is rather conservative in deciding what is really a viral inclusion body among a multitude of candidate particles. Also, raw suspensions may contain a high number of alkali-soluble particles of nonviral origin, particles which are

"accepted" as polyhedra with the electronic procedure. It appears that both counting procedures are quite unsatisfactory when the suspensions are extremely impure. For example, four paired counts, performed in our laboratory with one raw suspension of homogenized tissue containing nucleopolyhedra as well as a considerable proportion of insoluble particles in the size range of the polyhedra (17.1 percent of the gross particle count), gave consistently smaller numbers with the conventional microscopic procedure than with the electronic procedure. The microscopic counts averaged 51.4 percent of the corresponding electronic counts. It should be noted, however, that a disagreement of similar magnitude existed when microscopic counts of polyhedra in raw suspensions were performed by two independent observers, as exemplified in table 4!

Table 4.--*Two sets of microscopic counts of nucleopolyhedra from raw suspensions of tissue homogenates (six different preparations obtained from larvae of Hemerocampa pseudotsugata)*^{1/}

Preparations	Polyhedra per mg. dry weight ($\times 10^6$)		Count no. 2 as percent of count no. 1
	Count by observer no. 1	Count by observer no. 2	
A	60	30	50
B	60	18	30
C	50	23	46
D	52	21	40
E	50	21	42
F	48	26	54

^{1/}

The two observers worked independently in two separate laboratories, using essentially similar microscopic counting procedures.

We believe that polyhedron counts of suspensions prepared with raw tissue homogenates are unreliable, and that the biological assay of such suspensions provides a more consistent estimate of the potency of such preparations (e.g., LD₅₀ in nanograms of preparation per gram of larval weight). Microscopic as well as electronic polyhedron counting procedures are accurate and reliable when the inclusion-body suspensions are relatively clean.

Polyhedron-Size Distribution

The mean size of nucleopolyhedra from various species of insects has been determined with optical and electron microscopes by a number of investigators. The measurements were usually limited to relatively small samples (50 to 100 inclusion bodies).

The circuitry of the model B Coulter counter permits all particles of a size between two selected threshold levels to be counted and registered on glow-type decade counters. The upper and lower threshold discriminators screen out particle counts above and below a predetermined size. By means of this circuitry it is possible to determine accurately size distributions of large samples from a population of particles.

The size distributions of four different batches of nucleopolyhedra obtained from larvae of *Hemerocampa pseudotsugata* are illustrated in figure 3. The data relevant to the four samples are presented in table 5.

Table 5.--*Sample size, mean volume, and volume range of four nucleopolyhedron preparations, as determined with the Coulter transducer*

Preparation number	Number of polyhedra sampled	Mean volume of polyhedra, μ^3	Percent particles in volume (μ^3) range indicated
17	5,337	5.5	98.1 (1.6 to 10.6)
18	10,376	4.5	95.2 (1.6 to 10.6)
19	2,560	5.6	97.7 (1.6 to 10.6)
20	13,480	3.9	99.0 (0.9 to 9.3)

Electron microscope measurements are frequently standardized by means of a linear reference; e.g., a grating replica with 28,800 lines per inch. The calibration of our Coulter counter was based on the volume of spheres of monodisperse polystyrene latex sized by electron microscopy (average particle diameter 2.96 microns; standard deviation 0.016 micron). Mr. R. B. Addison, of our Forestry Sciences Laboratory, measured 50 particles of this latex suspension, using a Philips EM-200 microscope and a grating replica (28,800 lines per inch) as a primary standard. The microscope was used with only one condenser and with the lowest usable beam intensity in order to keep irradiation of the specimen to a minimum. The mean diameter of this sample was consistent

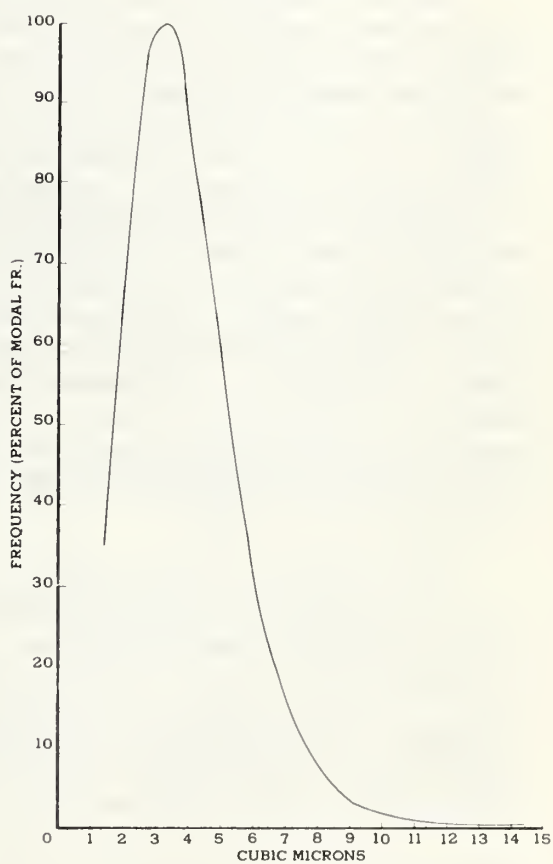
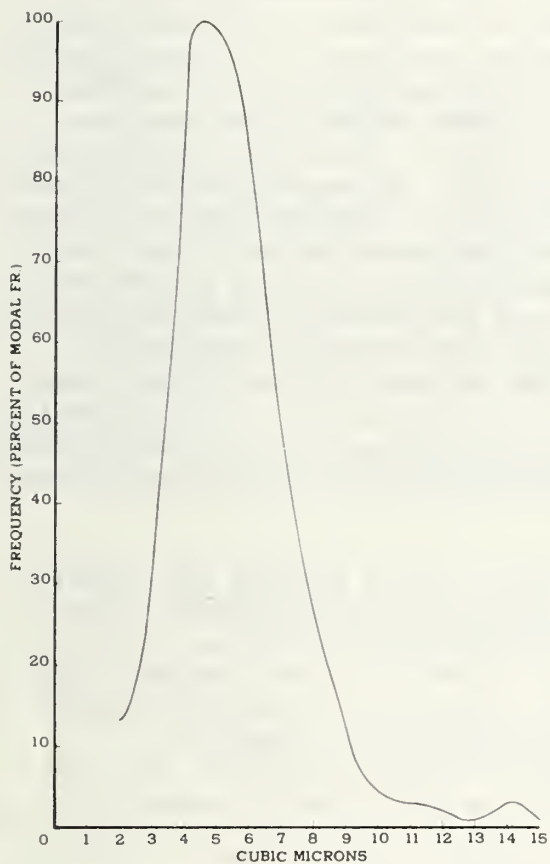
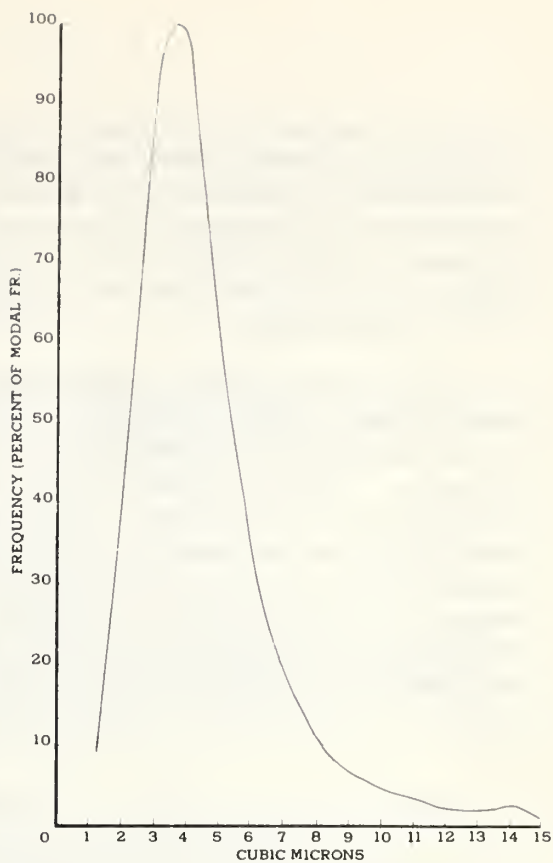


Figure 3.--Relative polyhedron size distributions (modal frequency = 100) of four different purified preparations. The values on the x-axis are polyhedron volumes in cubic microns.

with that quoted by the manufacturer. Electron microscopic measurement is, at present, the best method available for the primary standardization of particle size distributions (Harvey and Marr 1966). Measurements of arrays of spheres with a phase contrast optical microscope (Kubitschek 1961; Bradford and Vanderhoff 1966) also gave values which did not deviate from the mean diameter estimate provided by the manufacturer.

A comparison of our polyhedron samples with the polyhedra measured by Morris (1963) can only be approximate, since the solid shape of the nuclear inclusion bodies of *Hemerocampa pseudotsugata* is very irregular. Morris found that the average "diameter" of 100 polyhedra measured was 1.5 microns, with a range of 1.2 microns to 2 microns. When allowance is made for the irregular shape of the polyhedra, this "diameter" is not inconsistent with the size of the polyhedra of our sample 20 (see table 5). However, a definitive comparison between samples should be based on identical sizing methods, equipment, and calibration standards to limit the sources of errors inevitably present in all indirect measuring procedures.

Summary

Coulter counts of nucleopolyhedra in purified suspensions and in suspensions containing varying amounts of impurities (up to 7.9 percent in the size range of the inclusion bodies) gave results in good agreement with counts performed with the traditional microscopic counting chamber. Since the sample size is very large in the electronic procedure, the relative standard errors (percent of mean) were reduced by a factor of 10 or more. Paired counts with raw suspensions of inclusion bodies (tissue homogenates containing 17.1 percent particles other than polyhedra in the size range of the inclusion bodies) showed poor agreement between the electronic and microscopic counting procedures. On the other hand, a similar disagreement was detected when two independent observers performed counts using the same procedure (microscopic technique). Polyhedron counts of suspensions containing large amounts of impurities (such as raw tissue homogenates) gave unreliable results by electronic as well as microscopic procedures. In such cases, biological assay remains the only reliable method for the determination of infectivity titers.

The circuitry of the model B Coulter counter permits one to count all particles of a size between two selected threshold levels. Thus it is possible to establish size frequency distributions of batches of nucleopolyhedra based on large numbers of particles (as compared with the 50 or 100 particles conventionally measured by electron microscopists). Four batches of polyhedra from one insect species (*Hemerocampa pseudotsugata*) were found to have similar mean particle volumes and volume frequency distributions when sized by means of a

30-micron aperture. The mean volumes of the polyhedra varied between $3.9 \mu^3$ and $5.6 \mu^3$. The Coulter transducer was calibrated with monodisperse polystyrene latex.

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